Article

Oxidative Stress and Ca^{2+} Release Events in Mouse Cardiomyocytes

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ABSTRACT Cellular oxidative stress, associated with a variety of common cardiac diseases, is well recognized to affect the function of several key proteins involved in Ca^{2+} signaling and excitation-contraction coupling, which are known to be exquisitely sensitive to reactive oxygen species. These include the Ca^{2+} release channels of the sarcoplasmic reticulum (ryanodine receptors or RyR2s) and the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Oxidation of RyR2s is found to increase the open probability of the channel, whereas CaMKII can be activated independently of Ca^{2+} through oxidation. Here, we investigated how oxidative stress affects RyR2 function and SR Ca^{2+} signaling in situ by analyzing Ca^{2+} sparks in permeabilized mouse cardiomyocytes under a broad range of oxidative conditions. The results show that with increasing oxidative stress Ca^{2+} spark duration is prolonged. In addition, long and very long-lasting (up to hundreds of milliseconds) localized Ca^{2+} release events started to appear, eventually leading to sarcoplasmic reticulum (SR) Ca^{2+} depletion. These changes of release duration could be prevented by the CaMKII inhibitor KN93 and did not occur in mice lacking the CaMKII-specific S2814 phosphorylation site on RyR2. The appearance of long-lasting Ca^{2+} release events was paralleled by an increase of RyR2 oxidation, but also by RyR-S2814 phosphorylation, and by CaMKII oxidation. Our results suggest that in a strongly oxidative environment oxidation-dependent activation of CaMKII leads to RyR2 phosphorylation and thereby contributes to the massive prolongation of SR Ca^{2+} release events.

INTRODUCTION

Cellular oxidative stress is increasingly recognized as a substantial contributor to the pathology of a wide range of diseases affecting cardiac muscle. Quite few studies have examined consequences of oxidative stress with particular focus on Ca^{2+} signaling and excitation-contraction (EC) coupling, because many Ca^{2+} signaling proteins are known to be exquisitely susceptible to oxidative posttranslational modifications (for reviews see (1–3)). In particular, the sarcoplasmic reticulum (SR) Ca^{2+} release channel (ryanodine receptor type 2 (RyR2)) was found to undergo an array of functional changes after redox modifications of cysteine residues (3). In general, under moderately and severely oxidizing conditions the RyR channels become more sensitive to cytosolic Ca^{2+} (4), which may lead to aberrant and arrhythmogenic Ca^{2+} release events occurring in diastole (e.g., Ca^{2+} waves). In addition, it has recently been reported that the Ca^{2+} calmodulin-dependent protein kinase II (CaMKII) can be converted into the active form by redox modification (5). This occurs after oxidation of methionine residues in the regulatory domain of CaMKII. Because RyR2s can be phosphorylated by CaMKII, this indirect pathway could also contribute to abnormal RyR2 behavior during diseases involving oxidative stress (6,7).

In the heart, the major sources of reactive oxygen species (ROS) include NAD(P)H oxidases (NOXs (8–10)), xanthine oxidase (XO (11)), and mitochondria (12,13). In several models of slowly progressing cardiac diseases, NOXs are overexpressed and exhibit a high degree of activity, thereby contributing to oxidative stress (e.g., after myocardial infarction, during hypertrophy and heart failure, dystrophic cardiomyopathy, for review see (14)). During the progression of such diseases Ca^{2+} signaling during excitation-contraction (EC) coupling may be altered, becoming abnormally sensitive to Ca^{2+} triggers (15,16). This may arise from changes of RyR2 Ca^{2+} sensitivity. During later stages of heart disease, the high RyR2 sensitivity may lead to long-lasting Ca^{2+} sparks, substantial diastolic SR Ca^{2+} leaks, ultimately contributing to the decline of SR Ca^{2+} content (together with the often reduced SERCA expression) (17), followed by smaller Ca^{2+} transients and weaker contractions (7,18,19). Experimentally, this RyR2 hypersensitivity could sometimes be normalized in vitro by compounds that neutralize or scavenge ROS or inhibitors of CaMKII. In vivo studies showed that treating animals with ROS scavenging compounds, such as *N*-acetyl cysteine or polyphenols (epigallocatechin gallate), delayed disease progression, lending further support for the notion that oxidative stress may be critically involved in the pathology of some cardiomyopathies (20). In addition, pharmacological and/or genetic inhibition of CaMKII phosphorylation

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of RyR2s improves cardiac performance in ROS-triggered dystrophic cardiomyopathy (16,21,22).

The ratio of reduced to oxidized glutathione in the cytosol ([GSH]/[GSSG]) is frequently used as an indicator and determinant of oxidative stress in different tissues, because it is a major cellular redox buffer in mammalian cells. In a typical healthy cardiac cell the overall ratio of [GSH]/[GSSG] determined with biochemical assays is around 30:1 (18). This ratio was found to be significantly reduced in various cardiac diseases, including dystrophic cardiomyopathy and heart failure (18). Of note, this ratio is an average value determined from all subcellular compartments, including the cytosol, mitochondria, SR, and other organelles. The cytosol itself is normally much less oxidized than some intracellular organelles, most notably the SR. Recent estimates of cytosolic ratios with a redox-sensitive genetic probe indicate [GSH]/[GSSG] ratios of several hundreds or even thousands to one (23). However, during several cardiac diseases, mitochondrial function will ultimately deteriorate, leading to permeability pore transitions and mitochondrial depolarization, which can be followed by apoptotic or necrotic cell death (13). Mitochondrial depolarization is accompanied by a highly localized but extremely strong burst of [illegible] in the vicinity of a particular mitochondrion. Such [illegible] can be detected with confocal imaging and ROS-sensitive fluorescent chemical or genetic indicators and [illegible] referred to as superoxide flashes (2[illegible]). The precise extent of oxidative stress generated within these local ROS bursts is presently unknown, [illegible] much higher than the average cellular redox potentials determined with biochemical techniques.

To analyze the role and importance of altered redox potentials and the ramifications of these strong ROS flashes on the function of the RyR2s, we examined Ca^{2+} sparks as an indicator of RyR2 activity using permeabilized ventricular myocytes. In this experimental system, we could apply a wide range of well-defined strongly oxidizing conditions by adjusting the [GSH]/[GSSG] ratios. Such conditions would obviously not be survivable by intact cardiomyocytes, if present throughout the entire cytosol.

In these experiments we found, in pronounced oxidative environments, dramatic changes in morphology of the Ca^{2+} sparks, most notably the appearance of long and very long-lasting Ca^{2+} release events, which only terminated after hundreds of milliseconds. The appearance of these events ultimately resulted in a modest depletion of SR Ca^{2+} content. These conditions also resulted in oxidative activation of CaMKII and phosphorylation of the RyR2s at the serine 2814 site. The number of the long-lasting Ca^{2+} release events was reduced by inhibitors of CaMKII and they were not observed in transgenic mice with an ablated S2814 phosphorylation site on the RyR2s. Thus, extremely oxidative conditions, as they can for example be observed as superoxide flashes near mitochondria, may be able to affect RyR2 gating and SR Ca^{2+} release. Abnormal SR Ca^{2+} release may be, in addition to its arrhythmogenic potential, relevant for activation of downstream signals triggering cellular damage (e.g., calpain activation, apoptosis) and progression of the disease.

MATERIALS AND METHODS

Isolation of cardiomyocyte[illegible]

After cervical dislocation of 4–6 month[illegible] C57BL/10 mice hearts were quickly removed and cardiac ventricular myocytes were enzymatically isolated using retrograde cardiac perfusion on a Langendorff apparatus with solutions (37°C) containing collagenase as previously described (26). All animal handling and experimental procedures were carried out following the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals (1996) and were approved by the Swiss State and Federal Veterinary Offices.

Cell permeabilization

Cells were permeabilized by exposure to the saponin β-escin (20 μM), added to a relaxing solution that contained: 110 mM K^+ aspartate, 10 mM EGTA (reduced to 0.1 mM for Ca^{2+} wave experiments), 10 mM phosphocreatine, 5 units/ml creatine phosphokinase, 4% dextran (mol. wt. [illegible]), 3 mM $MgCl_2$, 3 mM K-ATP, 50 μM fluo-3, 10 mM HEPES, and 3 mM GSSG+GSH. Concentration of free Ca^{2+} was adjusted to 60 nM, free Mg^{2+} to 1 mM, pH was 7.2, temperature 20–22°C. The time course of loading of the cytosolic space with fluo-3 was carefully followed to rapidly remove the β-escin before it could compromise the function of intracellular organelles. On average the loading procedure lasted around 15 min. The timing for oxidative stress application was kept similar for all cells.

Solutions with various oxidative strengths

The solutions contained a total of 3 mM GSH+GSSG. The composition was as follows: 2.99 mM GSH + 0.01 mM GSSG (ratio 300:1); 2.86 + 0.14 mM (ratio 20:1) 2.25 + 0.75 mM (ratio 3:1); 1.5 + 1.5 mM (ratio 1:1); 0.5 + 2.5 mM (ratio 1:5). The ambient oxygen pressure determined the P_{O2} (~150 mmHg). To estimate SR content, Ca^{2+} transients resulting from a puff of 10 mM caffeine applied to the permeabilized cells were recorded.

Voltage-clamp experiments

For estimates of SERCA activity Ca^{2+} transients were triggered with L-type Ca^{2+} currents in myocytes under whole-cell patch-clamp conditions. Cells were dialyzed with pipette filling solutions containing [GSH]/[GSSG] at ratios of 300:1 and 1:5. Solutions were identical to those used for permeabilized cells, but without dextran (see above). After a prepulse protocol to load the SR, a depolarization to 0 mV lasting 200 ms was applied from a holding potential of −50 mV, to activate L-type Ca^{2+} current and trigger a Ca^{2+} transient.

Confocal Ca^{2+} imaging

Fluo-3 was excited with the 488 nm line of an argon-ion laser, attenuated to 50 μW. Fluorescence was detected at >500 nm with a laser-scanning confocal microscope (μRadiance, Bio-Rad Laboratories, Hemel Hempstead, UK) operating in linescan mode (500 lines/s) along the longitudinal cell axis, using a 60×, 1.2 N.A. water immersion objective. The frequency

and spatiotemporal parameters of the Ca^{2+} sparks were determined from linescan images using ImageJ analysis software with the SparkMaster algorithm (27). Ca^{2+} spark frequencies are expressed as the number of recorded sparks per second and per 100 μm of scanned distance, spark width as full width at half-maximal amplitude (in μm), duration as full duration at half-maximal amplitude (FDHM in ms). Confocal linescan images are shown as pseudoratiometric images obtained by linewise normalization (28).

Biochemical assay

Mouse ventricular samples were sonicated in the radio-immunoprecipitation assay lysis buffer (1× TBS, 1% P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide supplemented with phenylmethylsulfonyl fluoride, protease inhibitor cocktail and Calyculin A (Santa Cruz, CA)). Protein concentration in a sample was determined with an Eppendorf photometer using bovine serum albumin (BSA) as a standard. For immunoblotting, 40 μg of protein lysate per sample was denaturated in 4× SDS-PAGE sample loading buffer and loaded to 4% (for RyR2) or 10% (for CaMKII) SDS-polyacrylamide gel, respectively. Blots were then transferred in nitrocellulose membrane (Bio-Rad, CA) at 15V for 16–18 h at 4°C (for RyR2) and at 90V for 90 min at 4°C (for CaMKII), blocked with 1% BSA in TBS and Tween (TBS: 20 mM TRIS-HCl, 200 mM NaCl, 0.6% Tween 20, pH 7.5) for 1 h and probed for proteins of interest. Levels of RyR2, phosphorylated-RyR2, CaMKII, and oxidized-CaMKII were assessed using specific primary antibodies: anti-RyR2 (1:500, Thermo Scientific, Waltham, MA), anti-phosphorylated-RyR2-2808 (1:2000, Badrilla, Leeds, UK), anti-phosphorylated-RyR2-2814 (1:500, Badrilla, UK), anti-CaMKII and anti-oxidized-CaMKII (5) (1:500, both w supplied by Dr. M. Anderson, Iowa City, IA), and appropriate ho adi peroxidase-conjugated secondary antibodies. Membranes were w d 3 times for 15 min in TBST after primary antibodies and s ndary anti incubation, respectively. The chemiluminescence cted an quired onto MultiImage light cabinet (Alpha otech an Lean CA). For Oxyblots, cardiac SR samples (1 were pr ed accord to previous literature (29), and then resusp nded ff ES, 500 mM sucrose, pH7.4) and incuba with H_2 0 mM) or GSSG (5 mM) for 30 min at room tempe . After reacti e samples were centrifuged at 110,000 × *g* fo pellets from entrifugation were resuspended in buffer. C dation RyR2 was detected by anti-DNP antibody (Oxyblot, M pore, Billeric A).

Statistical a sis

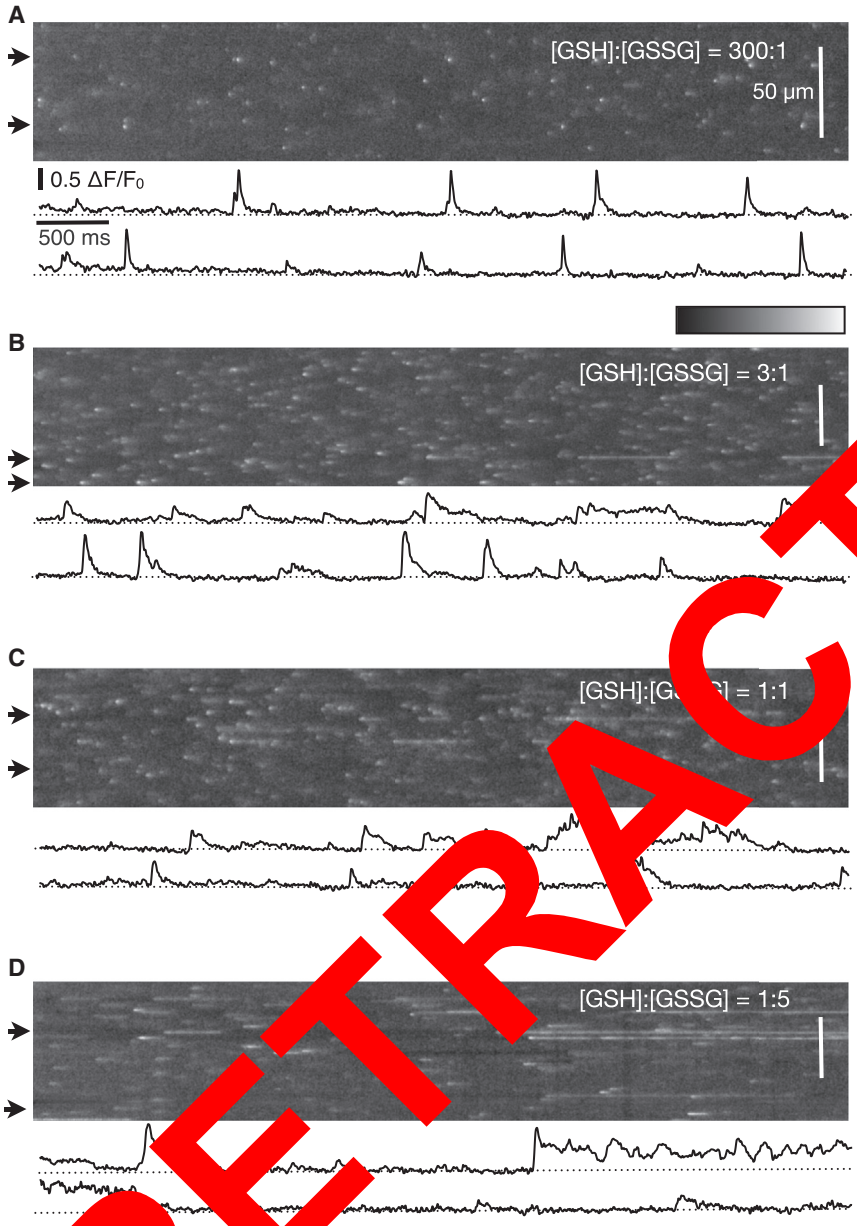
Results are as m stand error of the mean. Except in the experime tu sgenic mice the number (*n*) of sparks was high an 500 nts for all e rimental groups. N for other types of experi s are e figure legends, all data were collected from at least t ence o olations. Statistical significance was evaluated by paired paired Student's *t*-test. A *p*-value of <0.05 was considered significant. S ficance is indicated by ** on the graphs.

RESULTS

Changes of Ca^{2+} spark parameters under oxidative stress

In the first series of experiments, we tested the hypothesis that the oxidized cytosolic redox potential would result in higher Ca^{2+} spark frequencies as a manifestation of elevated RyR2 open probabilities. Permeabilized mouse cardiomyocytes were placed for ~3 min (sufficient to reach a steady state of spark frequencies) in solutions with [GSH]/[GSSG] ratios ranging from 300:1 to 1:5, while the total concentration of [GSH]+[GSSG] was kept constant at 3 mM. The ratio of 300:1 is in the range of normal cytosolic conditions (23). Fig. 1, *A–D*, shows confocal linescan images of fluo-3 fluorescence recorded under increasingly oxidizing conditions. The linescan images shown were processed by linewise fluorescence normalization (i.e., pseudoratiometric images (2 ected fluorescent profiles from the regions mark with a s are shown below the linescan images. he near n al cytosolic environment ([GSH]/[GSSG] ra of 300:1 pontaneous Ca^{2+} sparks were obs ed at a f en of around 25 sparks per 100 μm anned ne and ond (Fig. 1 *A*), which is compara o findings in previous studies, where neither SH GSSG been added (30). A modest ch e of the ra o 1 did not make a difference for Ca^{2+} spark para ters. However, when changing to a more ative solution ([GSH]/[GSSG] ratio of 3:1 1 *B*), th ark frequency increased by ~40%, duration (FDHM) by 20% and the amplitude by 20% (Fig. 2 *A*). S rk rise times did not change markedly. These odificatio of the spark parameters were associated with derate crease in estimated SR Ca^{2+} load, defined as the de of a Ca^{2+} transient induced by local application of 10 mM caffeine (see Fig. 3). We also carried out ol experiments with lower and higher concentrations of GSH and GSSG (but constant redox potential) to exclude direct pharmacological effects of these compounds on the RyR2s (data not shown).

Previous studies found that even limited oxidative stress, imposed as decreased [GSH]/[GSSG] ratios in the cytosolic space, can lead to oscillatory instabilities of the mitochondrial membrane potential and ultimately to mitochondrial depolarization by a mechanisms also called ROS-induced ROS release (24,31). Thereby, the depolarizing mitochondria generate a burst of ROS which results in a highly oxidized but spatially confined domain in their vicinity (superoxide flashes) (25). In an attempt to mimic the redox potentials prevailing in such domains, we decreased the [GSH]/[GSSG] ratios of the experimental solution further, to cover a large range of redox potentials. This corresponds to the range of ratios over which the RyRs have been found to respond in ryanodine binding assays (4). Stronger oxidation with [GSH]/[GSSG] ratios of 1:1 and 1:5, respectively, led to gradual decreases in spark frequency and amplitude (Fig. 2 *A*) along with a reduction in SR Ca^{2+} load, when compared to a ratio of 3:1 (Fig. 3). In parallel, the duration of Ca^{2+} sparks was progressively increased. This was caused by the appearance of long-lasting Ca^{2+} release events (here defined as events with durations more than an arbitrary threshold of 70 ms, Fig. 2, *B–D*). At very oxidized conditions extremely long-lasting Ca^{2+} release events (with durations >200 ms) started to appear, often extending beyond the end of the linescan recording (Fig. 1 *D*). Overall, the total number of events (sparks



Fl Ca2+ ease events recorded from perme ed e cardiomyocytes in different oxidizing ions. (*A*) In control conditions spontaneous individual Ca^{2+} sparks can be solved. (*B*) In more oxidized conditions, more + sparks appeared, they exhibited a longer du tion and a few very long-lasting Ca^{2+} release events could be detected. (*C*) These changes became more pronounced as the oxidative stress increased. (*D*) In highly oxidative conditions, there was a large number of long-lasting Ca^{2+} release events, whereas the number of regular sparks was reduced. Look-up table and scale bars apply to all traces.

plu ng-l ts) remained approximately constant in ox e conditions.

Long-lasting events of Ca^{2+} release are inhibited by antagonists of CaMKII

A number of reports have described an increased frequency and duration of Ca^{2+} sparks in cardiomyocytes upon activation of CaMKII (32–34). Transgenic mice expressing a constitutively phosphorylated RyR2 channel (S2814D) showed elevated Ca^{2+} spark frequencies leading to SR Ca^{2+} depletion and slightly prolonged Ca^{2+} sparks (35). Moreover, studies at the single-channel level revealed that CaMKII phosphorylation of RyR2 dramatically increases its open probability and open time (36,37). Therefore, we next examined whether the morphology of Ca^{2+} release events present under strong oxidative conditions can be normalized using an exogenous CaMKII inhibitor. Fig. 4 *A* shows representative linescan images of fluo-3 fluorescence under severely oxidized conditions (control, GSH]/[GSSG] ratio of 1:5), and after addition of the CaMKII inhibitor KN93 (5 μM) or its inactive negative control compound KN92 (5 μM). In the presence of KN93 the appearance of long-lasting Ca^{2+} release events was minimal, although they were obvious under control conditions and in the presence of the negative control compound KN92. Fig. 4 *B* summarizes changes in event duration resulting from KN93 and KN92 at [GSH]/[GSSG] ratios of 3:1 and 1:5. In both conditions, inhibition of CaMKII-dependent phosphorylation by KN93 significantly reduced

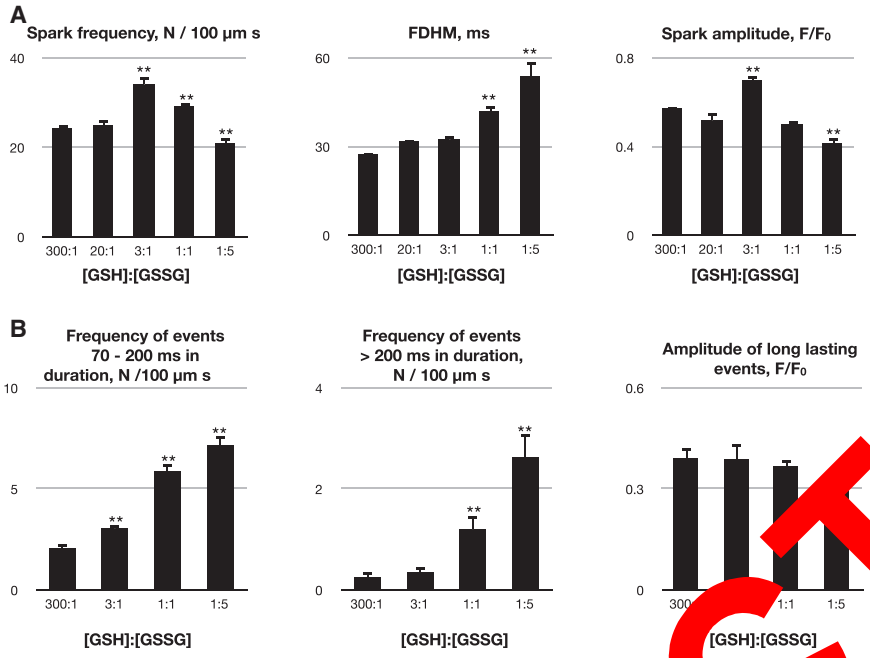


FIGURE 2 Analysis of Ca^{2+} spark and release event parameters. (*A*) Spark frequency, duration (FDHM), and am… Although a moderate oxidative …ss did not … spark parameters significa… more sever… oxidative stress increase… the fre…cy of spark… and their amplitude. …r numbe… ampli… again declined in… oxidative con… the mean duration …ually i…ased. (*B*) … number of long (70–2…) …very-lon… (>200 ms) events gradually incr… with m… evere oxidative stress. Their amplitu… m… declined, similar to the amplitude of sh… ents.

the number of long-lasting events, thereby slig… creasing the number of shorter events. KN92 did no… the long-lasting release signals, confirming that KN9… acting via CaMKII inhibition. Taken toget… this sug… that oxidative stress primarily leads to a …atio… of CaM… and consequent CaMKII-mediate… hosph…lation RyR2. The latter in turn increases R… o… …ity (and presumably open time), …ich be…es manifest in the appearance of long spa…

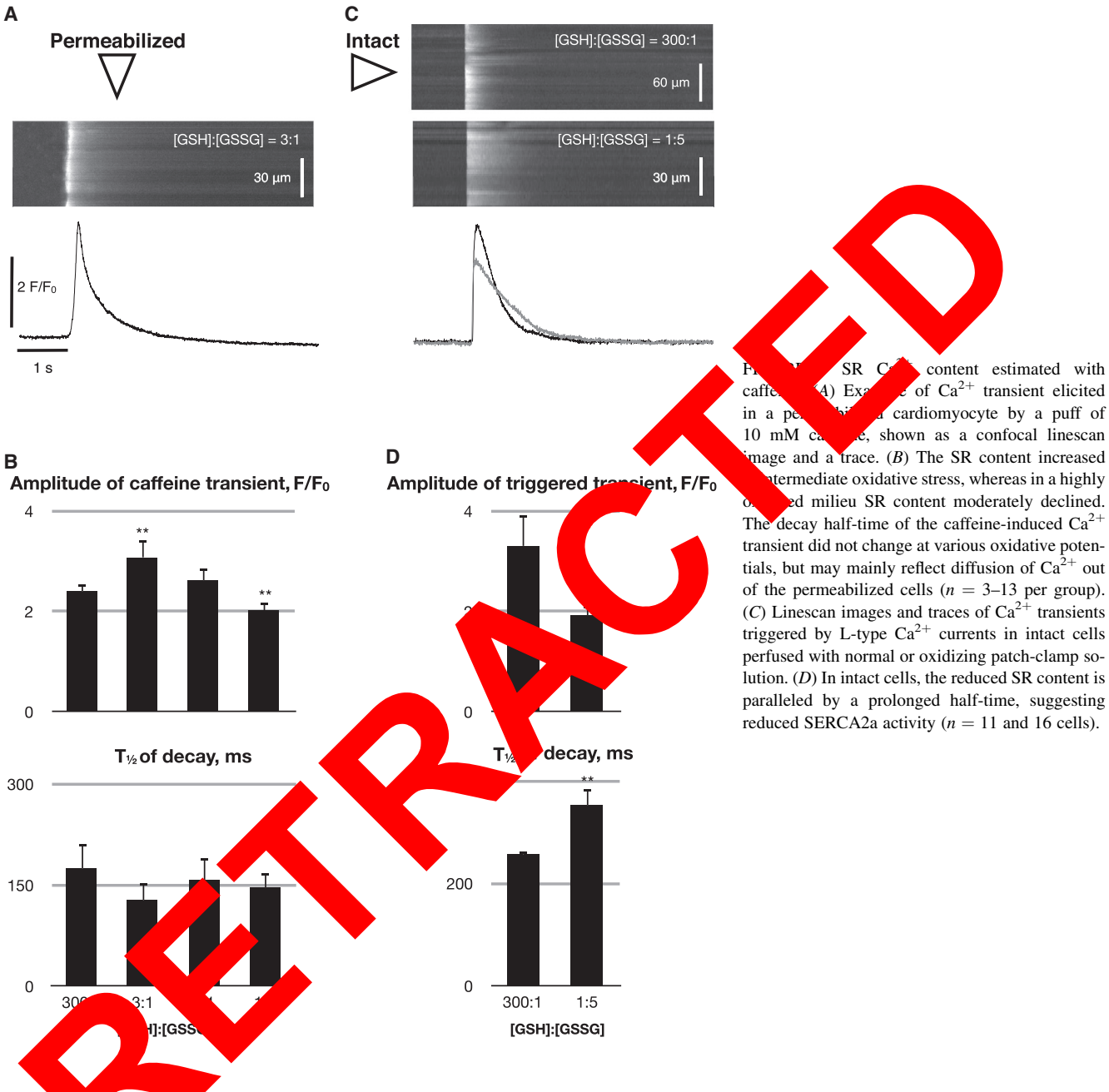
Long-lasting eve… of Ca^{2+} r…se are absent in cardiomyocyte… with ablated Ca…KII-phosphoryla…n sit… 2814 on RyR2

Additional evidenc… a CaM…-dependent mechanism of oxidati… tion o… Ca^{2+} release was obtained in experi…nts wi… mouse c…iomyocytes harboring RyR2s in wh… seri… has been replaced by alanine (RyR-S2814… 29). This substitution made the RyR2s resistant to CaMK… hosphorylation, but did not change SR Ca^{2+} content. Fig… *A* shows linescan images of Ca^{2+} release events in two different redox conditions in myocytes isolated from wild-type (WT) and RyR-S2814A mice. In the strongly oxidizing solution ([GSH]/[GSSG] ratio of 1:5), the presence of long-lasting Ca^{2+} release events is apparent in WT cells, but not in those lacking the CaMKII phosphorylation site on RyR2. The analysis of Ca^{2+} sparks in Fig. 5 *B* reveals that, as expected, there was no significant difference in spark parameters between the two groups of cells in moderately oxidized media ([GSH]/[GSSG] ratio of 3:1). However, under very strong oxidation ([GSH]/[GSSG] ratio of 1:5) the duration of the events significantly increased in cel… WT but not S2814A mice, as a result of the …ppearance of long-lasting events. In fact, events with dura… longer than 70 ms were completely absent in S2814A cells, even in strongly oxidizing conditions. In summary, the findings obtained in the RyR-S2814A cells corroborated the results obtained in the presence of the CaMKII blocker KN93 and confirmed the notion that the oxidative environment leads to long-lasting Ca^{2+} release events mainly via CaMKII activation (5) and subsequent RyR2 phosphorylation at serine 2814. This result also makes the participation of other channels, such as the $InsP_3$ receptor channels, unlikely.

Because sensitization of the RyR2s for Ca^{2+} may also increase the propensity for arrhythmias triggered by Ca^{2+} waves, we analyzed Ca^{2+} waves in permeabilized cardiomyocytes incubated in solutions with a [GSH]/[GSSG] ratio of 300:1 and 1:5 (Fig. 6). This experimental situation is of course very different from an in vivo event of a superoxide flash, where only a small microdomain becomes exposed to very strong oxidative stress. These measurements revealed a reduced occurrence of Ca^{2+} waves, but a significantly increased Ca^{2+} wave velocity.

RyR2s are phosphorylated at CaMKII but not protein kinase A sites

To obtain direct support for the hypothesis that RyR2 channels are phosphorylated at CaMKII sites in response to oxidative stress, we carried out a series of biochemical studies illustrated in Fig. 7. The degree of CaMKII oxidation, as well as protein kinase A (PKA) and CaMKII-dependent phosphorylation status of the RyR2s under different



F... SR C... content estimated with caffe... A) Ex... e of Ca^{2+} transient elicited in a pe... bi... cardiomyocyte by a puff of 10 mM ca... e, shown as a confocal linescan image and a trace. (*B*) The SR content increased ...termediate oxidative stress, whereas in a highly o... ed milieu SR content moderately declined. The decay half-time of the caffeine-induced Ca^{2+} transient did not change at various oxidative potentials, but may mainly reflect diffusion of Ca^{2+} out of the permeabilized cells (n = 3–13 per group). (*C*) Linescan images and traces of Ca^{2+} transients triggered by L-type Ca^{2+} currents in intact cells perfused with normal or oxidizing patch-clamp solution. (*D*) In intact cells, the reduced SR content is paralleled by a prolonged half-time, suggesting reduced SERCA2a activity (n = 11 and 16 cells).

redox c... ions were quantified using immunoblotting techniques. ...greement with this view, incubation of cardiac ventricular tissue with GSSG (5 mM) or H_2O_2 (10 mM) significantly increased the amount of oxidized CaMKII normalized to total CaMKII (Fig. 7, *A* and *B*). In addition, the same conditions also significantly increased the degree of RyR2 phosphorylation at the CaMKII S2814 site, but not at the PKA site S2808 (Fig. 7, *C* and *D*).

Because most of the changes of the spark parameters appeared to depend on the activity of CaMKII, we assessed the degree of RyR2 oxidation by performing an OxyBlot assay (Fig. 8). These results indicate that RyR2 becomes indeed more oxidized in the presence of GSSG, to an extent that is not significantly different from that observed in H_2O_2, which served as a positive control experiment.

DISCUSSION

Oxidative stress

Imbalance of the cellular redox system is recognized to lead to a plethora of changes in cardiomyocyte function (38). Oxidative stress accompanies a wide range of cardiac diseases, including conditions involving hypoxia, ischemia, inflammation, mechanical overload, and heart failure (2). In cardiomyocytes, several pathways have been found to

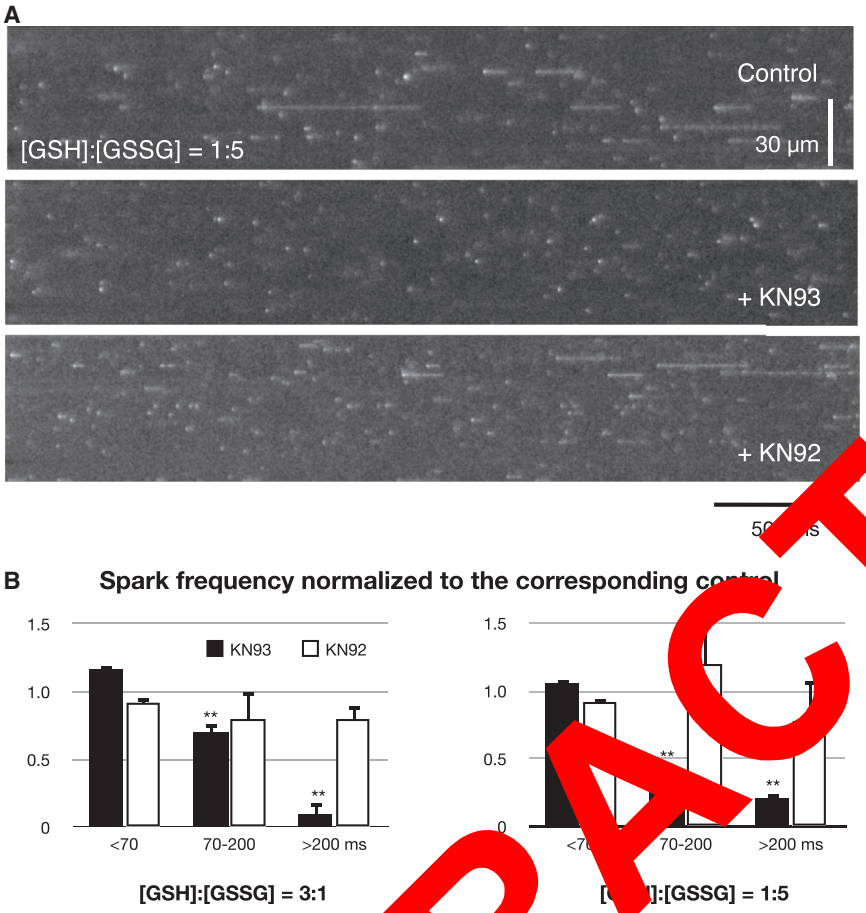


FIGURE 4 The appearance of long-lasting Ca^{2+} release events is controlled by CaMKII-dependent pathway. (*A*) KN93 completely suppressed the appearance of long-lasting release events in highly oxidized environment. In control and in presence of KN92, a negative control for KN93, no significant appeared. (*B*) Analysis of the long and long-lasting events in two different oxidative conditions. KN93 prevented the appearance of long events, whereas KN92 had no effect. Values were normalized to the corresponding values obtained under control conditions (no drugs applied).

be particularly sensitive to oxidative stress, including Ca^{2+} signaling, hypertrophic signaling and the oxidative stress defense pathway itself (31). In several of these pathologies oxidative stress is an important aggravator and drives disease progression (16). The main cellular sources of ROS in cardiomyocytes are NADPH oxidases (NOXs) located in the sarcolemma, the xanthine oxidases (XOs (11)) and the mitochondria (12).

Several proteins involved in cardiac SR Ca^{2+} cycling have been found to be exceptionally sensitive to changes in the redox environment (1,5,39). ROS can affect the gating properties of such Ca^{2+} channels or the activity of transporters and enzymes. However, it has remained largely unknown how this ROS sensitivity might manifest itself on the molecular level. For example, the function of RyRs (1,39) and CaMKII (5) were found to be very susceptible to redox modifications by ROS. Unwanted activation of this tandem of proteins by ROS could be especially critical, because each of them could further activate the other. CaMKII will phosphorylate (or hyperphosphorylate) the RyR2s, among many other proteins, increasing their open probability, whereas Ca^{2+} released by the RyR2s will further activate the CaMKII. With the given arrhythmogenic potential of diastolic SR Ca^{2+} release via hypersensitive RyR2s, such a condition may be extremely dangerous in the presence of preexisting alterations of RyR2 Ca^{2+} sensitivity, such as in channels harboring arrhythmogenic mutations. This can occur, despite the autoregulatory features of Ca^{2+}-induced Ca^{2+} release, which are expected to attenuate steady-state effects of isolated changes of RyR2 Ca^{2+} sensitivity (17). Based on this rationale, both the RyR2s and CaMKII have been proposed to be potential drug targets to treat Ca^{2+}-induced arrhythmias, either with RyR2 stabilizers (40,41) or CaMKII inhibitors (42).

General relevance of findings

It is established that both, RyR2s and CaMKII can become activated by redox modifications during oxidative stress, although not necessarily with the same sensitivity. Our results indicate that the combination of RyR2 oxidation and CaMKII activation (with subsequent RyR2 phosphorylation) leads to dramatic alterations of Ca^{2+} signaling in the microdomain of the T-tubule/SR junction (a.k.a. couplon), where EC-coupling takes place. However, it is surprising that in our experiments inhibition of CaMKII or removal of the CaMKII-dependent phosphorylation site

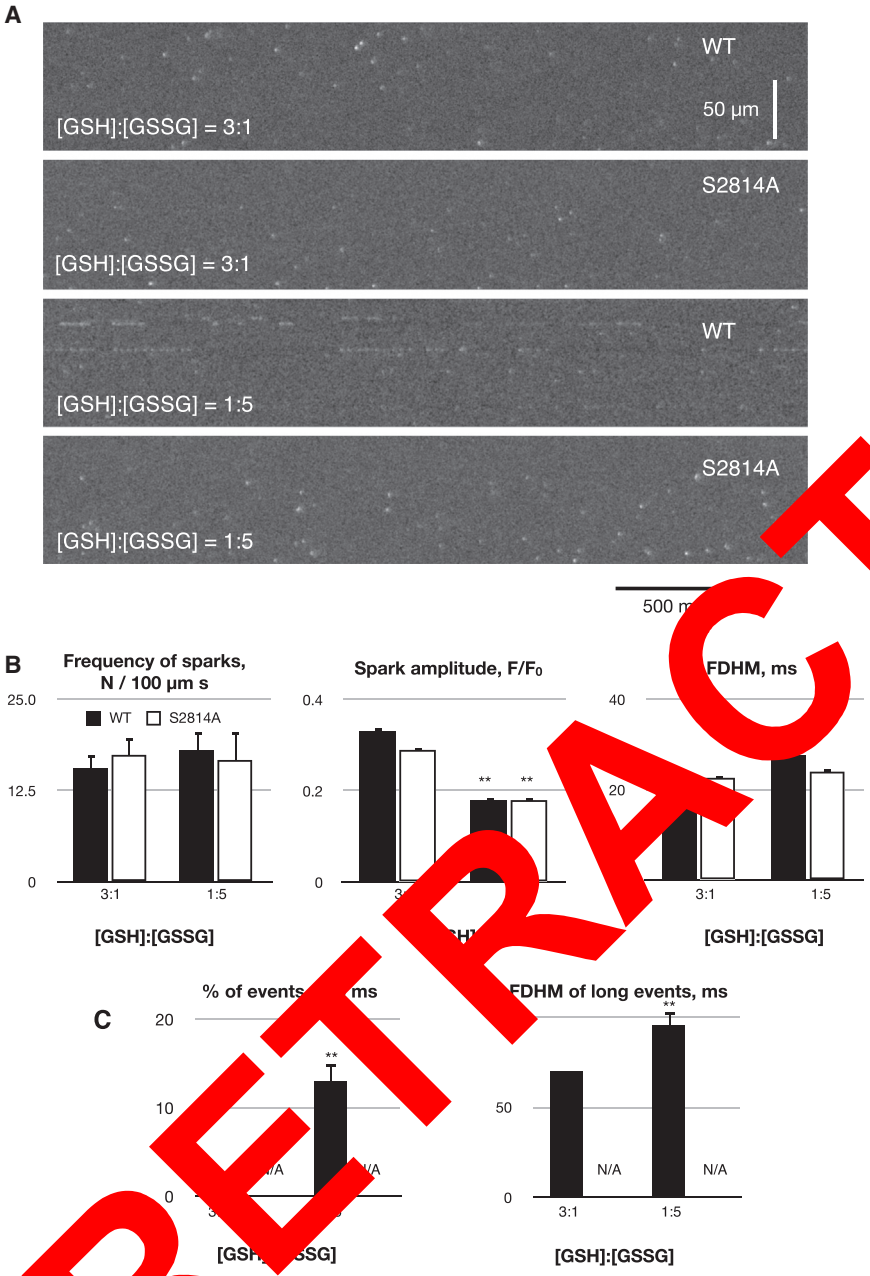


FIG 5 Ca^{2+} release events in cells lacking the S2 Ca phosphorylation site on the RyR2. (*A*) Although there were no spark differences in sparks in mildly oxidative conditions GSH]/[GSSG] of 3:1), long-lasting release events appeared with [GSH]/[GSSG] of 1:5, but only in WT cells, not in S2814A cells. (*B*) This is reflected in a prolongation of the mean event duration (FDHM) only seen in WT cells. (*C*) This mainly resulted from the appearance of long-lasting events (>70 ms) under oxidative conditions in WT cells (14% of all events). Note that events longer than 70 ms were not observed at all in S2814A cells (N/A; total sparks (*n*): 150 and 136 in WT, 169 and 195 in S2814A).

on the R s can largely prevent the occurrence of these abnormally g Ca^{2+} release events. Thus, it appears that the oxidation-dependent CaMKII phosphorylation of RyR2 phosphorylation plays the major role, whereas direct RyR2 oxidation has less severe consequences. This apparent imbalance between direct and indirect mechanisms may be partly related to the experimental conditions and could be quite different in vivo. As it has been reported before, the ambient partial pressure of oxygen is quite high in isolated cardiomyocytes, compared to that physiologically prevailing in tissue. Thus, under ambient experimental conditions some of the cysteines on RyR2 may not be available for oxidation, whereas the oxidation-sensitive methionine residues on CaMKII site are still available (5,43), even though we could biochemically detect at least some additional RyR2 oxidation under strongly oxidative conditions. However, based on our findings we cannot discount the possibility that in vivo oxidative stress will modify RyR2 function directly. In any case, the Ca^{2+} release events observed here had a much longer duration than what was found when CaMKII was activated in the absence of extra oxidative stress (33,34,44). Thus, RyR2 oxidation, although by itself not changing the sparks in situ, may have a permissive function by amplifying gating changes induced by CaMKII-dependent phosphorylation. This may occur, for

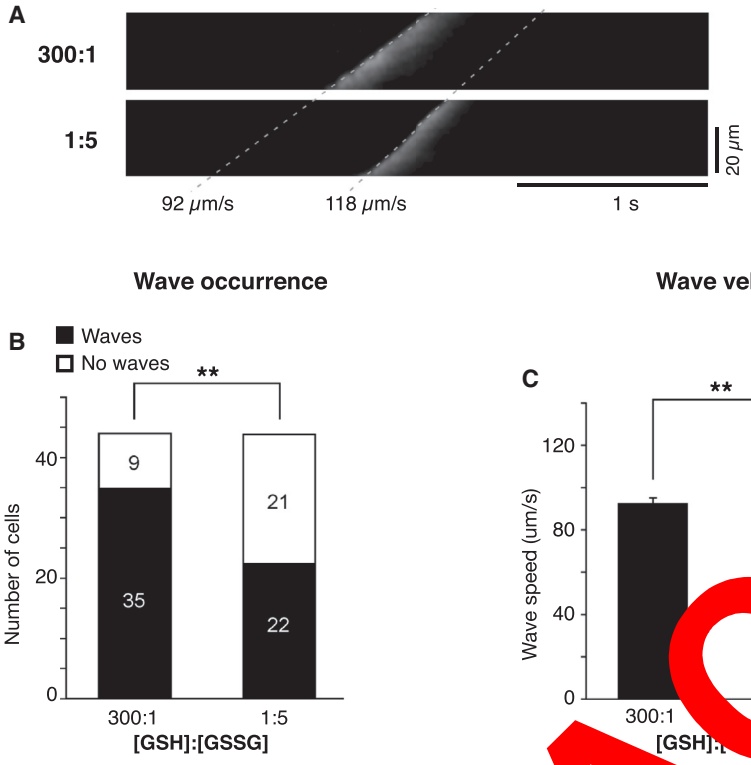


FIGURE 6 ...lysis of Ca... ave occurrence and velocity ...ermeabilize... myocytes. (*A*) Repres...ive Ca...ves in n...al and oxidized con...ns. Dotted lin...re...asted to the wave-f...to visu...wave sp... (*B*) Wave occurrence ...redu...n oxidized conditions. (*C*) Statistical an... Ca^{2+} w...peed revealed faster waves in an...ized e...onment ($n = 32$ (300:1) and $n = 19$ (...ectively).

example, by increases of eventless or quarky SR ...2 release (45). Therefore, it appears that tw...nslati... modifications of RyR2, phosphory...on an...oxidati... cooperate to increase RyR2 open ...abil...ibly in an additive way. Related ...erva... were recently reported in isolated pig ...rdiomyocy... after rapid pacing. In these experi...n the large ...2+ signals in the microdomain of the dy... in combination with ROS generated loc... by NAD(P)...idase, led to functional changes ...ouple... RyRs. The...e were manifest as higher spark ...enci... and better EC-coupling synchronization (46).

At intermediate levels of oxidative stress we observed a slight increase in the SR Ca^{2+} content. This may contribute to the higher spark frequency and spark amplitude and could be the result of CaMKII-dependent PLB phosphorylation and ROS-induced SERCA2a stimulation, which may occur at moderate oxidation of the protein. Several studies indicate that S-glutathionylation stimulates SERCA2a, whereas irreversible oxidation of cysteine 674 reduces its function (47,48). As oxidation became stronger, the frequency of long-lasting release events increased, finally representing a relevant SR Ca^{2+} leak. This leak, together with the oxidative SERCA2a inhibition, in turn resulted in a tendency of the

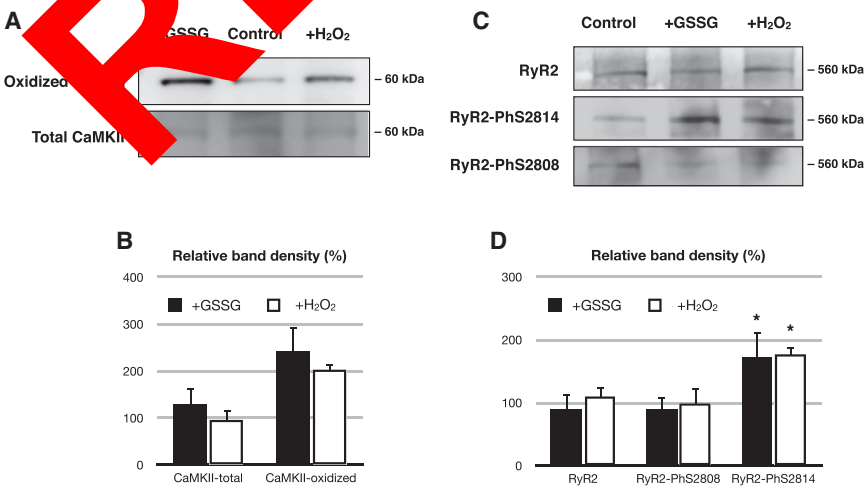


FIGURE 7 Biochemical quantification of CaMKII oxidation and RyR2 phosphorylation. (*A* and *B*) Immunoblots and summary of total and oxidized CaMKII. Band intensities recorded from $n = 3$ samples treated with 5 mM GSSG or 10 mM H_2O_2 are presented as a percentage of increase compared with values obtained in samples without treatment. Both interventions resulted in a significant increase in the oxidized form of CaMKII. (*C* and *D*) Immunoblots and summary of phosphorylated RyR2. As previously mentioned, band intensities treated with GSSG or H_2O_2 are presented as a percentage of increase compared with values obtained in samples without treatment. Whereas normalized intensity of CaMKII-dependent immunoreactivity significantly increased in samples treated with either GSSG or H_2O_2, the value did not change significantly for the PKA site.

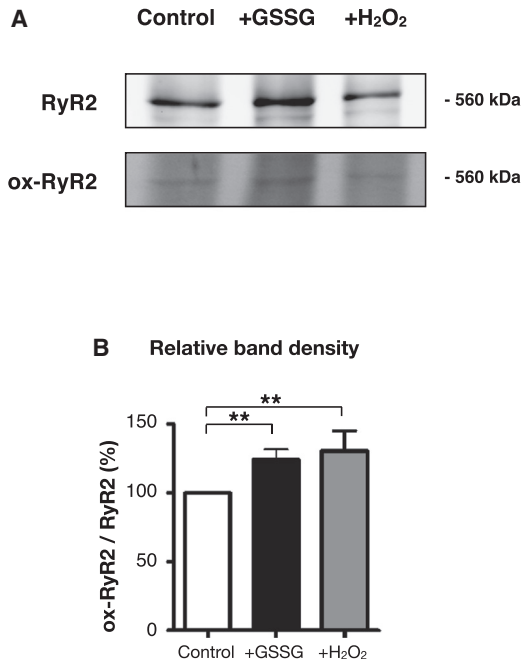


FIGURE 8 Biochemical assay of RyR2 oxidation. (*A*) OxyBlots of RyRs were carried out in control condition, in the presence of 5 mM GSSG and in the presence of 10 mM H_2O_2 ($n = 4$ samples). (*B*) In GSSG, the level of detected RyR2 oxidation, normalized for total RyR2, showed a significant increase in the presence of GSSG. This extent of oxidation was not significantly different from that in H_2O_2.

SR Ca^{2+} to decline and in a reduction of the number of sparks. Ca^{2+} sparks. Indeed, we observed concomitant reduction of SR Ca^{2+} content and slowing of Ca^{2+} transient decay (as an estimate of SERCA2a activity) in intact cells perfused with a strongly oxidizing patch pipette solution ([GSH]/[GSSG]: 1:5; see Fig. 3).

In the permeabilized cells, Ca^{2+} waves were quite rare. In strongly oxidizing solutions, only a few waves could be observed, presumably because of the reduced SR Ca^{2+} load, as Ca^{2+} waves seem to be mainly initiated by elevations of [Ca^{2+}]inside the SR (49). Interestingly, under these conditions the propagation velocity of these waves were significantly inconsistent with an elevated cytosolic Ca^{2+} sensitivity of the RyR2s.

Mechanisms underlying long-lasting Ca^{2+} release events

CaMKII-dependent phosphorylation has been reported to increase the open probability of RyR2s in the lipid bilayer by increasing their Ca^{2+} sensitivity. Although in some studies it prolonged open times in the millisecond range (37,50), very long openings lasting several seconds were observed as well (36). In addition, the RyR2s can open into long-lasting subconductance states. In principle, a single RyR2 exhibiting an extremely long opening could entrain a long-lasting SR Ca^{2+} release event by repeatedly activating neighboring RyR2s within the same cluster of channels (51,52). Similarly, multiple and repeated reopenings of several RyR2 channels within one couplon could occur, resulting from the changed Ca^{2+} sensitivity and channel gating properties. Instead of a continued or repeated activation of a couplon (which we cannot exclude based on our results), the underlying mechanism may be related to altered SR Ca^{2+} release termination (53,54). This could be mechanistically linked to enhanced RyR2 sensitivity for cytosolic Ca^{2+}, but could also arise from other changes of RyR function. In this context it is interesting to note that micromolar caffeine concentrations, which also sensitize the RyR2s for Ca^{2+}, do not seem to increase the duration of Ca^{2+} sparks (55). The precise mechanism responsible for termination of a release of channels is not yet known, but several possibilities have been proposed. This includes Ca^{2+}-dependent inactivation of the RyRs (56), depletion of the Ca^{2+} store sensed by calsequestrin (57), and stochastic attrition (58), whereby Ca^{2+} release eventually terminates when all RyRs are simultaneously closed. Recent concepts of spark termination suggest that the low sensitivity of the RyR2s (micromolar) for activation by Ca^{2+} in combination with the declining SR Ca^{2+} content and dwindling release flux could conspire to interrupt this mutual channel activation within the couplon, leading to termination by pernicious attrition (59,60). However, when the sensitivity of the RyR2s for activation by Ca^{2+} in the dyadic cleft is abnormally high, such as after phosphorylation by CaMKII and RyR2 oxidation, Ca^{2+} release termination by any of these mechanisms could be delayed and thus Ca^{2+} release signals would be prolonged. The number of actually open RyR2 channels may be less than during a regular spark, either because of gating changes induced by oxidation and/or CaMKII-dependent phosphorylation or because of a lower Ca^{2+} concentration in the dyadic cleft, as the release flux decreases (61). During the long duration of the release events, the Ca^{2+} concentration inside the junctional SR of a release site will reach a low steady-state level, governed by the balance of SR Ca^{2+} release flux and diffusion of Ca^{2+} inside the SR to the couplon (62–64). As long as this intra-SR Ca^{2+} level sustains a release flux that keeps the dyadic Ca^{2+} concentration above cutoff for termination, release can continue. This situation is mechanistically attractive as it could also explain the long-lasting Ca^{2+} release events observed under a variety of circumstances (65), such as, for example, from the nuclear envelope, which is part of the SR but has a very large volume close to the release site (66). There, the decline of local $[Ca^{2+}]_{SR}$ would be small, which could preclude or at least delay termination.

Limitations of the study

Imposing various redox potentials on cardiomyocytes will obviously affect many signaling systems, including a few that are relevant for Ca^{2+} cycling. Thus, there are many

possibilities for confounding side effects of oxidative stress. In this study, we used Ca^{2+} spark detection to specifically examine the function of the RyRs and changes resulting from the altered redox environment. Pharmacological tools and a transgenic animal approach indicate that CaMKII and the related S2814 phosphorylation site on the RyR2s play an important role in the way the RyR2s change their function, which is our main conclusion. However, this type of analysis will not address changes of other Ca^{2+} signaling components, such as SERCA or the eventless SR Ca^{2+} leak, both of which could indirectly contribute to our observations. The same applies for the condition of room temperature and the ambient oxygen pressure, which is higher than the one prevailing in intact cardiac muscle.

As with any other study involving transgenic animals, there is always the possibility of adaptive changes. In the case of the S2814A mice with ablated CaMKII-dependent phosphorylation site on the RyR we know that these animals have no phenotype alterations at baseline (29). Of particular importance for this study is the observation that the SR Ca^{2+} content is unchanged under baseline conditions.

CONCLUSIONS

In cardiomyocytes, oxidative conditions result in [illegible] increase of the Ca^{2+} spark frequency, whereas strong oxidative stress leads to dramatic changes of [illegible] Ca^{2+} release event duration. This occurs mainly via [illegible] of CaMKII, by methionine oxidation of [illegible] regulatory subunit. Subsequently, CaMKII phosphorylates [illegible] the CaMKII-specific serine 2814 site, whereas phosphorylation at the PKA site (2808) remains unchanged. [illegible] some cardiac diseases and pathologic states such a strong oxidative environment is likely to prevail in the microdomain of mitochondrial superoxide flashes, generated by a burst of mitochondrial ROS triggering intermittent openings of the mitochondrial permeability transition pore. Such bursts of ROS are thought to be produced, for example, during ischemia-reperfusion and are known to accompany apoptotic and necrotic cell death. The extremely long duration of ROS-induced Ca^{2+} release events leads to SR Ca^{2+} depletion because of the resulting Ca^{2+} leak/uptake imbalance. The long duration is also consistent with a sensitization of the RyR2s for cytosolic Ca^{2+} and with delayed Ca^{2+} release termination. However, based on these observations other modes of RyR2 sensitization, such as by SR luminal Ca^{2+}, cannot be excluded and could contribute to the long Ca^{2+} release duration.

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